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A BODIPY-Based Probe for the Selective Detection of Hypochlorous Acid in Living Cells

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Reactive oxygen species (ROS) are essential for a wide range of biological and pathological events.[1] During infection and inflammation, the phagocytic leukocytes, including neutrophils, monocytes, and macrophages generate reactive oxygen species (ROS) to kill invading bacteria and pathogens.[2] Among ROS, hypochlorous acid ($HOCl/OCl^-$) is a highly reactive oxygen species produced from hydrogen peroxide (H_2O_2) and chloride ions (Cl^-) by the enzyme *myeloperoxidase* (MPO), which is secreted by activated neutrophils.[3] Although hypochlorous acid plays important roles in the human immune-defense system, overproduction of ROS in living organism has detrimental effects on biological molecules, including nucleic acids, lipids, and proteins, resulting in the inhibition of various protein functions, and contributes to the progression of numerous human diseases, such as atherosclerosis, cancer, cardiovascular diseases, and rheumatoid arthritis.[4]

Despite its importance in human health and disease, not as much is known about the mechanism of action and specific roles of HOCl in living systems in comparison with other ROS, owing to slower progress in the development of suitable probes. Several fluorescence probes for the detection and visualization of HOCl in living cells have recently been developed on the basis of the strong oxidizing properties of HOCl.[5–7] HOCl-induced oxidation reactions were employed in the design of fluorescent probes in which the fluorescence properties were regulated by the conversion of the spirocyclic form of rhodamine fluorophores into their ring-opened form,[5] or through photoinduced-electron-transfer processes.[6] To facilitate practical applications of such probes, next-generation designs should emphasize higher analyte selectivity, limit susceptibility to autooxidation, and avoid demanding multistep syntheses. Herein, we report the facile synthesis and properties of a new boron-dipyrromethene (BODIPY) dye bearing a methylthioether group, and its biological application as a highly sensitive and selective 'turn-on' fluorescent probe for HOCl in living cells. We envisioned that the oxidation of thioethers into sulfoxides in the presence of HOCl could act as a sensing trigger to modulate the optical properties of molecular probes. We chose a BODIPY-dye scaffold owing to their desirable features that include high absorption coefficients, high fluorescence quantum yields, excellent photostability, and versatile chemistry.[8] Their tolerance to spontaneous autooxidation and photobleaching is equally advantageous under bioimaging conditions. Substitution of the BODIPY core at the 2- and 6-positions is known to greatly affect its photophysical properties. In particular, the introduction of electron-donating groups at these positions results in bathochromically shifted absorption and emission bands, and substitution with heavy atoms is known to cause marked decrease in fluorescence quantum yields.[9]

To introduce the desired methylthioether group at these nucleophilic positions, we relied on the known reaction of nucleophilic π-systems with activated dimethyl sulfoxide reagents.[10] Thus, the methylthio-substituted BODIPY probe **2**, is easily prepared in a single step starting from **1** by an electrophilic substitution reaction according to Scheme 1. The formation of **2** likely occurs through the reaction of **1** with in-situ generated chlorodimethylsulfonium chloride, which reacts either as a chlorinating or a thiomethylating agent, and provides probe **2** in high yield (74%). This simple synthetic procedure is also applicable to other BODIPY derivatives (See the Supporting Information).

We investigated the photophysical properties of the new methylthio-BODIPY **2** in various solvents (See the Support-

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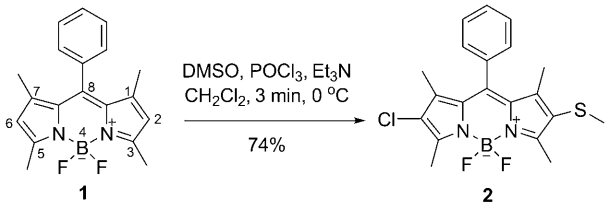
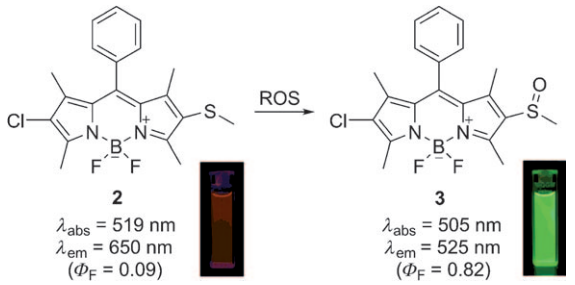


Scheme 1. Synthesis of methylthio-BODIPY **2**. DMSO = dimethyl sulfoxide.

ing Information). Probe **2** shows an absorption maximum at a wavelength comparable to that of precursor **1**, but exhibits a broad red-emission band with an unusually large Stokes shift. In aqueous solution, probe **2** displays absorption and emission maxima at 519 nm and 650 nm, respectively. The emission spectrum of **2** is affected by solvent polarity (see the Supporting Information, Table S1), which implies a degree of internal charge transfer character associated with these electronic transitions. As expected, probe **2** presents a reduced fluorescence-quantum yield (Φ_F 0.09) relative to the parent BODIPY **1** (Φ_F 0.62).[11]

As the introduction of the electron-donating methylthioether substituent at the 2-position dramatically reduces the quantum yield and red-shifts its emission spectra, oxidation of the thioether into the corresponding electron-withdrawing sulfoxide was predicted to restore the quantum yield of the fluorophore and blue-shift its emission spectra, thus providing a dark-field turn-on signal in the presence of HOCl. To confirm this hypothesis, the expected oxidation product **3** was prepared by oxidation of **2** and fully characterized (see the Supporting Information). Gratifyingly, conversion into the sulfoxide was accompanied by hypsochromic shifts in the absorption (λ_{abs} 505 nm) and emission (λ_{em} 525 nm) maxima that were evident to the naked eye (Scheme 2). Moreover, **3** exhibits an excellent fluorescence quantum yield (Φ_F 0.82) in aqueous solution, as anticipated.



Scheme 2. Proposed sensing mode of probe **2**.

To evaluate the practical utility of probe **2** in the detection of HOCl ($pK_a = 7.6$), the pH dependence of the photophysical properties of **2** and **3** was investigated in a series of buffers with different pH values ranging from 3 to 9. These results (see the Supporting Information, Figure S4) indicate that the emission spectra of both compounds **2** and **3** are essentially pH-insensitive across this biologically relevant pH range whereas the absorption spectra of probe **2** are slightly red-shifted under acidic conditions (pH 3-6).

First, we measured the sensory response of probe **2** toward NaOCl in HEPES buffer (10 mM, pH 7.4, 0.2% DMSO). As shown in Figure 1, the activation of probe **2**

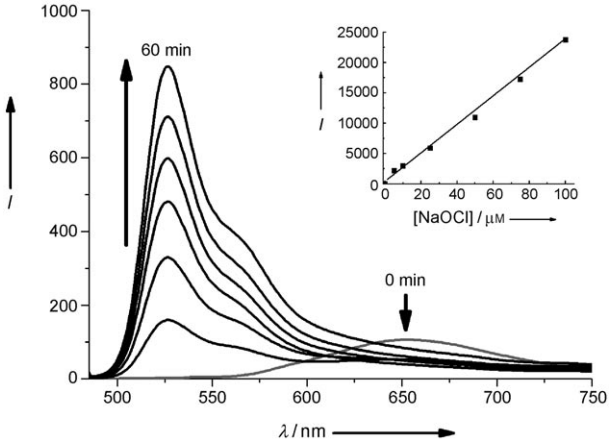


Figure 1. Fluorescence turn-on response of **2** (5 μM) to NaOCl (50 μM). The spectra were obtained every 10 min (0–60 min) after the addition of NaOCl. All data were obtained in HEPES buffer (10 mM, pH 7.4, 0.2% DMSO) at 25 °C. Excited at 465 nm. Inset: A linear correlation between emission intensity and concentrations of NaOCl. The emission intensity of probe **2** (5 μM) at 525 nm was determined after incubation at 25 °C for 1 h in presence of NaOCl.

after addition of NaOCl results in a decrease of emission intensity at 650 nm, together with the instant growth of the emission band of **3** at 525 nm. A remarkable increase in the fluorescence intensity was observed, depending on the concentration and reaction time (see the Supporting Information). Upon treatment with 10 equivalents of NaOCl, the solution of probe **2** already exhibited an approximately 88-fold increase in its fluorescence intensity within only 10 minutes. A linear correlation between the emission intensity and the concentration of NaOCl was also found (Figure 1, inset).

Figure 2 compares the reactivity of probe **2** toward various ROS. Probe **2** showed a positive response only in the

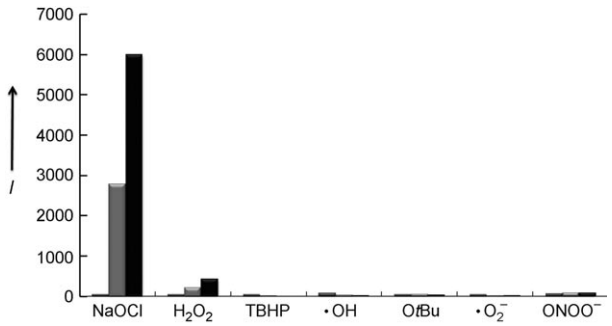


Figure 2. Relative fluorescence responses of **2** (5 μM) to various ROS (25 μM NaOCl, 25 μM H_2O_2, and 200 μM for others). Bars represent relative responses at 0, 30, and 60 min after the addition of each ROS. Emission signals was recorded at 525 nm (excited at 465 nm). All data were obtained in HEPES buffer (10 mM, pH 7.4, 0.2% DMSO) at 25 °C.

presence of NaOCl and H_2O_2, whereas no significant fluorescence signal at 525 nm was observed in presence of other ROS, such as *tert*-butyl hydroperoxide (TBHP), superoxide (O_2^-), hydroxyl radical (·OH), peroxynitrite ($ONOO^-$), and *tert*-butoxy radical (·O*t*Bu). The response of probe **2** also shows selectivity greater than 10:1 in favor of HOCl over H_2O_2.[12] LC-MS analysis of the test solutions confirmed that the major product of the reaction of probe **2** with either NaOCl or H_2O_2 was the expected oxidation product **3**. Hence, spectral changes observed in the presence of NaOCl or H_2O_2 origin from the oxidation of the methylthioether **2** into the sulfoxide **3** (Figure 3; also see the Supporting Information).

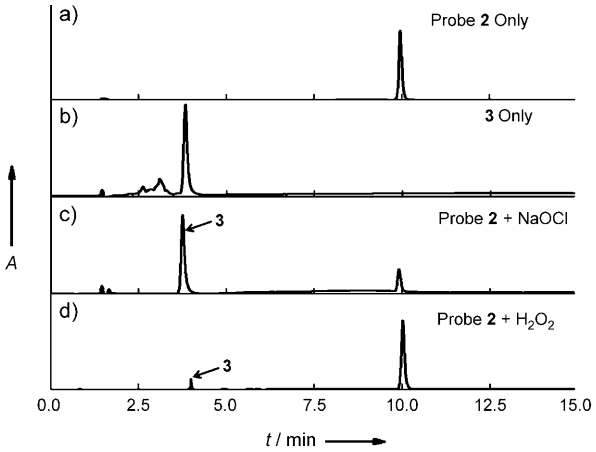


Figure 3. HPLC chromatograms of probe **2** a) without ROS treatment, c) after reaction with NaOCl for 60 min at 25 °C, d) after reaction with H_2O_2 for 60 min at 25 °C, and b) sulfoxide **3** only. The samples were analyzed by LC-MS with a linear gradient elution (eluent A/B = 20:80, A: deionised water with 1% formic acid, B: acetonitrile, flow rate 0.3 mL min^{-1}). The MW of the retention time at 10.0 min was 405.2, which corresponds to $[M+H]^+$ for probe **2** and MW of the retention time at 3.8 min is 421.1, which corresponds to $[M+H]^+$ for sulfoxide **3**. [**2**] = 5 μM, [NaOCl] = [H_2O_2] = 50 μM.

We next applied probe **2** to the imaging of HOCl in living RAW264.7 macrophages. Confocal microscopic images of probe-**2**-loaded macrophages that were treated with exogenous NaOCl for 20 minutes at 37 °C showed an increase in green fluorescence inside living cells whereas macrophages untreated with NaOCl showed negligible intracellular fluorescence (Figure 4 a,b). The fluorescence intensity within the cells increased linearly with the concentration of added NaOCl (see the Supporting Information, Figure S17 and S18). In the case of cells treated with 100 μM NaOCl, a more-than 5-fold increase in fluorescence intensity was observed compared to the control cells. The lower analytical responses observed for cell imaging experiments (5-fold) over solution assays (up to ca. 1000-fold) reflect the difficulty in the permeation of hypochlorous acid through the cell membrane given that the HOCl/$^-$OCl couple is, to a large extent, present in its anionic form at physiologically relevant pH.[13] In spite of these fundamental limitations, probe **2**,

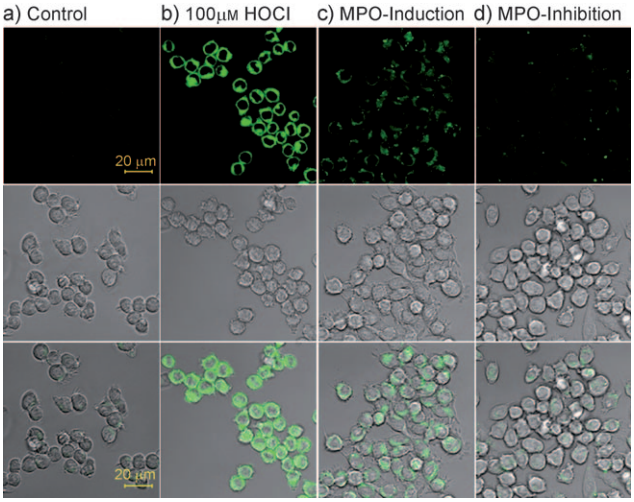


Figure 4. Relative confocal fluorescence images of living macrophages (RAW264.7) under different conditions with probe **2**. a) Macrophages were incubated with 5 μM probe **2** for 1 h at 37 °C and then imaged. b) Macrophage cells loaded with Probe **2** were incubated with 100 μM NaOCl for 20 min. c) Macrophage cells were stimulated with 1 μg mL^{-1} LPS for 24 h, further stimulated with 1 μg mL^{-1} PMA for 1 h, and incubated with 5 μM probe **2** for 1 h at 37 °C before being imaged. d) MPO inhibitor, 4-ABAH (final concentration = 100 μM) was co-incubated during PMA stimulation; the other procedures were the same. (top: fluorescence images, middle: bright-field images, bottom: merged images, Ex = 488 nm, Em = 505–550 nm).

which showed a 5-fold signal increase within 20 minutes in the presence of 100 μM HOCl, compares favorably with the only other probe for which comparable experimental cell-imaging data in RAW264.7 macrophages have been reported, a 4-aminophenyl fluorescein reporter,[7b] for which Chang and co-workers have found less than a 2-fold signal increase within 40 minutes upon treatment with 100 μM HOCl.[14]

Cell-viability assays confirm that probe **2** shows low cytotoxicity to macrophage cells over a concentration range from 5 to 50 μM (see the Supporting Information). Having established that probe **2** is cell-permeable, non-toxic, and able to measure HOCl levels in biological systems, we evaluated the potential utility of probe **2** for monitoring endogenously generated-HOCl at low signaling levels upon physiological stimulation. Macrophages and other phagocytic cells are known to produce low micromolar levels of H_2O_2 or other ROS during phagocytosis, or when stimulated by agents such as lipopolysaccharide (LPS) and phorbol myristate acetate (PMA).[15] Up to 80% of H_2O_2 generated is converted into HOCl/OCl^- in a reaction catalyzed by the *myeloperoxidase* (MPO) enzyme, which is localized in phagocytic leukocytes.[16] In addition, the production of MPO in the macrophage cells can be further induced upon stimulation.[16] RAW264.7 macrophage cells were incubated with stimulants to induce an immune response.

When LPS- and PMA-activated macrophage cells were incubated with probe **2**, a 2.2-fold increase of fluorescence intensity was observed in the cells, whereas no obvious fluo-

rescence was observed in the cells untreated with the stimulants (Figure 4c). Upon treatment with a MPO inhibitor[17] during stimulation, no obvious increase of fluorescence signal in the cells was observed (Figure 4d). These experiments demonstrate that probe **2** enables the visualization of both exogenous and endogenous HOCl in living cells.

In summary, we have shown that methylthio-BODIPY **2** is easily prepared from readily accessible precursors and constitutes a highly sensitive and selective fluorescence turn-on probe for HOCl in living cells. HOCl-selective probe **2** joins the expanding toolbox of ROS-specific probes[18] that can be applied to the challenging yet critically important elucidation of the roles of individual ROS in biological and pathological processes.

Experimental Section

Full experimental details and characterization data are given in the Supporting Information.

*Synthesis of Compound **2***

Dimethyl sulfoxide (1.8 mL, 22.5 mmol) and triethylamine (1 drop) were added to a stirred solution of **1** (100 mg, 0.31 mmol) in dichloromethane (1 mL) at room temperature. The resulting solution was cooled to 0 °C in an ice-water bath. Phosphorus oxychloride (40 μL, 0.46 mmol) was added dropwise to the solution via syringe, and the contents were stirred for 3 min at 0 °C. The solution rapidly turned from orange to dark-red. Following the removal of the solvent under reduced pressure, an excess of water (30 mL) was added to the reaction mixture. The crude red precipitate obtained by filtration was purified by column chromatography on silica gel using progressively more polar eluent, 10:1 to 5:1 hexanes/ethyl acetate, to afford **2** (92.5 mg, 74 %).

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[11] Quantum yields (Φ_F) of probe **2** and parent BODIPY **1** in various solvents are presented in the Supporting Information, Table S1.
[12] In preparative organic syntheses, the oxidation of thioethers into sulfoxides can be accomplished with many oxidizing agents, including peracids or hydrogen peroxide in the presence of various catalysts. Organic peroxides, such as *tert*-butyl hydroperoxide have also been widely used as stoichiometric oxidants in such transition-metal-catalyzed reactions. However, in the absence of catalysts, the reaction of hydrogen peroxides or alkyl hydroperoxides is sluggish in aqueous solutions at room temperature and physiological pH. By contrast, HOCl shows sufficient oxidizing power under these conditions for the oxidation to proceed, which helps rationalize the observed selectivity of probe **2**. See: a) E. L. Dane, S. B. King, T. M. Swager, *J. Am. Chem. Soc.* **2010**, *132*, 7758–7768; b) L. Kuhnen, *Angew. Chem.* **1966**, *78*, 937; *Angew. Chem. Int. Ed. Engl.* **1966**, *5*, 893; c) C. R. Johnson, D. McCants, Jr., *J. Am. Chem. Soc.* **1965**, *87*, 1109–1114; d) M. Madesclaire, *Tetrahedron* **1986**, *42*, 5459–5495.
[13] A slow internalization of hypochlorite is further supported by comparison with the unionized H_2O_2 ($pK_a = 11.6$). Whilst cells treated with 100 μM HOCl conserve a healthy morphology indicative of low internalized concentrations of the ROS, treatment with only 25 μM of the more cell-permeable H_2O_2 already causes significant cytotoxicity. Consequently, longer reaction times and/or higher concentrations of the analyte were required to compensate for the slow kinetics of internalization of hypochlorite into the cells.
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